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EFFECTS OF *COMBRETUM HERERONENSE* AND *CANTHIUM MUNDIANUM* water EXTRACTS ON PRODUCTION AND EXPRESSION OF INTERLEUKIN-4

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Abstract

Background: *Combretum hereroense* and *Canthium mundianum* are two plants commonly used by traditional healers in the Northern region of Limpopo, South Africa for the treatment of diarrhea and inflammation. In the present study, the effects of their water extracts on the production and expression of interleukin-4 by peripheral blood mononuclear cells (PBMC'S) from HIV positive and negative individuals was evaluated.
Materials and methods: Blood samples were collected from both HIV positive and HIV negative volunteers and were used for the purification of Peripheral blood mononuclear cells (*PBMC*). The PBMCs were cultured together with the water extracts after activation with phytohemagglutinin (PHA) for three days. Solid-phase sandwich ELISA (MABTECH) kit was used to detect IL-4 on un-stimulated and stimulated PBMC'S with phytohemaglutinin (PHA) and plant extracts, followed by the isolation of RNA using RNAesy Qiagen mini kit from the cells. Reverse transcriptase real time PCR was used to evaluate IL-4 gene expression by the cells.
Results: *Combretum hereroense* showed higher production of IL-4 at three different concentrations and a significant expression of mRNA with 4-fold amplification increase at 300µg/ml and 2-fold amplification increase at 20µg/ml. *Canthium mundianum* also showed increased production of IL-4 at 300µg/ml, but inhibited its production at 20µg/ml. Both extracts showed no expression at 50µg/ml. The response of the PBMCs from HIV negative individuals was more pronounced than that of HIV positive individuals who mostly increased production of IL4 at smaller concentrations unlike their HIV negative counterparts. Although in vitro studies do not necessarily predict in vivo outcomes, the plant extracts modulated the immune system by enhancing the production and expression of IL-4 in both HIV- and HIV+ individuals at different concentrations.
Conclusions: For the first time we have shown that the immunomodulatory effect of medicinal plants may depend on the clinical status of the individual. The present study revealed that the effect of the water extracts from the two plants on IL-4 expression and production is dependent on the microbiological state of the individual and is dose dependent. Further studies are needed to identify the active components in the extracts and also characterize the patients further for a better understanding of the mechanisms of action of these extracts.

Key words: *interleukin-4, peripheral mononuclear cells, HIV, ELISA, gene expression*

Introduction

Medicinal plants are an important aspect of the daily lives of many South Africans, and an important part of South Africa's cultural heritage. No fewer than 3 000 plant species are used for medicinal purposes in South Africa (Cogne et al., 2001; Vasisht et al., 2016). In general, about 350 species are used and traded. Unfortunately there is a lack of detailed documentation regarding the use of traditional medicinal plants in South Africa probably as a result of the urbanization and cultural changes that are taking place (McGaw et al., 2000). Presently, South Africa's contribution to world medicine includes the Cape aloe (Aloe ferox), buchu (*Agathosma betulina*) and devil's claw (*Harpagophytum procumbens*) (Morris, 2002). These medicinal plants are known to affect the human immune system and therefore increase the efficiency of the body to clear an infection (Altfeld et al., 2000; Nakada et al., 2002; Gholamnezhad et al., 2015). *Combretum hereronse* and *Canthium mundianum* are two medicinal plants used by the local Venda population as general tonic and for the management of gastro-intestinal infections. *Combretum hereroense* is also used as treatment for malaria while *Canthium mundianum* was reported to be used in the treatment of prostate cancer (Mabogo, 1990).

South Africa is also one of the countries which are increasingly threatened by infectious diseases such as HIV/AIDS and TB, which are developing multidrug resistance; therefore it is important to develop alternative and complementary approaches to circumvent the problems. (Norman, 2000; Plaeger, 2003). Cytokines play an important role in the modulation of immune system as they are

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produced by different types of cells and targeting the cytokines could be one of the logical approaches for prevention and treatment of these diseases (Nelson et al., 2003; Spelman et al., 2006). However, very few studies have been conducted in order to determine the effects of South African medicinal plants and Venda medicinal plants in particular on the immune system.

Interleukin-4 is a key cytokine regulating humoral and adaptive immunity that induces differentiation of naive helper T-cells (Th0 cells) to Th2 cells (Nelson et al., 2003). Upon activation by IL-4, Th2 cells subsequently produce additional IL-4. IL-4 stimulates activated B-cell and T-cell proliferation, and the differentiation of $CD4^+$ T-cells into Th2 cells (Rangarajan et al., 2000; Yamaguchi et al., 2016). It also induces B-cell class switching to IgE, B cell class switching to IgG4 and up-regulates MHC class II production (Goldsby et al., 2000). It decreases the production of Th1 cells, macrophages, INF-gamma, and dendritic cell (Delfan et al., 2015). The presence of IL-4 in extravascular tissues promotes alternative activation of macrophages into M2 cells and inhibits classical activation of macrophages into M1 cells (Nakada et al., 2002). An increase in repair macrophages (M2) is coupled with secretion of IL-10 and TGF-β that result in diminution of pathological inflammation (Ganz, 2012). Therefore, an increased production of IL4 can be used as a marker of the reduction of inflammation. Previous studies have shown that medicinal plants have effect on the production of IL4. For example *Nigella sativa* have an effect on the production of IL4 (Gholamnezhad et al., 2015).

Similarly, Jayaprakasam et al (2013) showed that constituents of an anti-asthma formulation decreased the production of IL4 as well as IL5 by murine TH2 cells. However, very few or no studies have been conducted on South African medicinal plants. Therefore, the present study determined the effects of medicinal plants extracts on the production and expression of IL-4 in vitro using peripheral blood mononuclear cells (PBMC'S).

Materials and Methods

Ethical consideration

Ethical clearance for this study was obtained from the Health and ethical committee of the University of Venda and permission to obtain blood samples from the hospitals was obtained from the Department of Health in Limpopo, Polokwane. Ethical clearance was also obtained from the Donald Fraser hospitals and Tshilidzini hospital.

Preparation of plant material

Plant material, (leaves, bark and roots) of *Combretum hereronse* (Voucher Number AS75) and *Canthium mundianum* (Voucher Number AS74) were collected from Malavuwe village Thulamela municipality Limpopo province. Validation of plant material was carried out at the herbarium of the Department of Botany, University of Venda, where the herbarium vouchers have been kept.

Preparation of extract

Because the traditional healers had indicated that they use water to prepare their medicine, we decided to prepare it with the same extractant in order to evaluate the plants as used traditionally. The plant materials were rinsed thoroughly with tap water to remove extraneous contaminants and chopped into small pieces and were oven-dried at 50ºC until stability of dry weight was observed, and then ground into powder with an electric-grinder. Extraction was carried out by sonication and macerating. An amount of 100g powdered plant materials was poured in stopper flasks containing 1000ml of distilled water. After filtration, the extracts were freeze-dried.

Blood collection

Blood samples were collected from HIV+ and HIV- patients at Donald Fraser hospital situated at about 20km from the University of Venda and at the University of Venda clinic by trained phlebotomists working at these institutions. Approximately 5ml of whole blood was collected into a vacutainer tube with EDTA (Ethylenediaminetetraacetic acid) and the tubes were inverted several times to ensure complete mixture of contents. The samples were transported to University of Venda Microbiology laboratory for analysis.

Isolation of white blood cells (peripheral blood mononuclear cells)

Blood was transferred to 15ml sterile centrifuge conical tubes and centrifuged for 10 minutes at 2000rpm and the plasma was transferred into cryotubes, and then stored at -80^{o}C. For PBMC isolation, a mixture of Blood/PBS, 1:2 dilutions was prepared and the tubes were inverted several times to ensure the complete mixture of blood and PBS. Briefly, the mixture was overlaid on 4ml ficoll-plaque and centrifuged at 1200rpm for 30 minutes without a break. Buffy coat was aspirated into a new 15ml centrifuge conical tube using 10ml pipettes and the volume was adjusted to 15ml mark with PBS and centrifuged at 1200rpm for 10 minutes. The supernatant was removed and the pellet was resuspended in 4ml ammonium chloride solution (lysis buffer). The mixture was incubated for 10 minutes at room temperature to allow the lysis of remaining red blood cells. The volume of the tubes were adjusted to the 15ml mark with PBS and centrifuged at 1200rpm for 10 minutes. The supernatant was removed and the cells were resuspended

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with 500µl of PBS and mixed gently. Cells were adjusted to the required number using heamocytometer by mixing 10µl of cells with 10µl of trypan blue to view cells on the microscope. Cells were aliquoted per tube into 15ml tubes and the volume was adjusted to the 15ml mark and centrifuged at 1200rpm for 10 minutes. The supernatant was removed and cells were resuspended in fetal calf serum with 10% DMSO and transferred to cryotubes and stored in the freezer at -80^{o}C until use.

Culturing of peripheral blood mononuclear cells

Culturing of PBMC was done according to Yu et al., (1989). Briefly, the cells were washed twice with RPMI media and were resuspended into a culture medium. About 0.5 ml of cell suspension was added into 24 wells tissue culture plate and additional 0.5 ml of medium was added, yielding a final volume of 1ml. PHA was added to the cells for activation after which, the plant extracts were added to different wells at three different concentrations including C1= 300µg/ml, C2= 50µg/ml and C3= 20µg/ml. Two wells tow which only PHA has been added and an equal amount of water, were included for each extract and were used as negative controls. The effects of the plant extracts on the cells were expressed as activity index and calculated as indicated below. Following the addition of the extracts the plates were incubated for 3 days at 37°C with 5% CO_2 100% humidity. After 3 days of culture, the supernatant was removed; particulates were removed from the supernatant by centrifugation and stored at -80^{o}C until used.

Evaluation of cytokine production by the enzyme-linked immunosorbent assay

The supernatant of the cultures including the negative control wells and the test wells containing three concentrations of the extracts were used for the ELISA test. The evaluation of IL-4 production was performed by solid-phase sandwich ELISA of Human IL-4 according to the manufacture's protocol (MABTECH AB, SWEDEN).

Total RNA purification from peripheral blood mononuclear cells

Total RNA was extracted from peripheral mononuclear cells treated with plant extracts after stimulation with phytohemagglutinin. Total RNA was extracted using the Qiagen RNeasy mini kit (Qiagen, Germany) according to the manufacture's protocol.

Reverse transcriptase-real time PCR

For the evaluation of PHA-inducible gene expression level, total RNA from cells (treated with the plant extracts as well as PHA stimulated or unstimulated) was used to prepare the cDNA using Reverse transcriptase polymerase chain reaction followed by real time PCR using the Maxima SYBER GREEN qPCR master mix (2x). The master mix was prepared by adding 10µl of SYBER GREEN, 0.6µl of forward and reverse primers of IL-4 and 5.8µl of nuclease free water. On each well 17µl of master mix was added and then 3µl of cDNA was also added to give a total volume of 20µl in each well. The PCR reaction was run for 45 cycles of 95ºC for 10 sec, 60ºC for 15 sec, and 72ºC for 10 sec following a pre-denaturation step at 95ºC for 10min and a final cooling step at 40^{o}C for 20sec. The primers used were IL-4 Forward: 5`-CCACGGACACAAGTGCGATAT-3` and IL-4 Reverse: 5`-CGTAACAGACATCTTTGCTGCC-3` previously described by Kowalczewska et al., (2014).

Data analysis

The level of IL-4 was measured from three HIV negative and two HIV positive donors by ELISA. Mean optical density from all the blood donors was calculated and used to determine the activity index of each plant extract on the expression of IL-4 using the following formula (Yeap et al., 2010):

$$\text{Activity index} = \{\underline{\text{OD plant extract} - \text{OD PHA}}\} \times 100$$
$$\text{OD PHA}$$

The Amplification fold for the real time PCR was determined by subtracting the control CT value from that of the plant extracts divided by the CT value of the control.

Results

The effects of the plant extracts on IL-4 production as determined by the ELISA method from HIV+ and HIV- donors.

The results obtained from ELISA indicated that cell culture supernatant from peripheral blood mononuclear cells cultured with *Combretum hereroense* (E1) and *Canthium mundianum* (E2) extracts showed different levels of IL-4 production in all the participants and it was dose dependent. Donor (A), the production of IL-4 was directly proportional with the increase in concentration for both extracts since high concentration yielded high production of IL-

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4. The production of IL-4 was similar to that of donor (A) in donor (B); however, at high concentration (300µg/ml) *C. hereroense* did not have any activity. Donor (C) behaved differently since IL-4 production was inversely proportional with the increase in concentration (high concentration yielded low IL-4 production) thereby inhibiting IL-4. HIV positive (D) showed similar results to donor (C), but only the extract from *Canthium mundianum* induced an increased production of IL-4 at all concentrations.

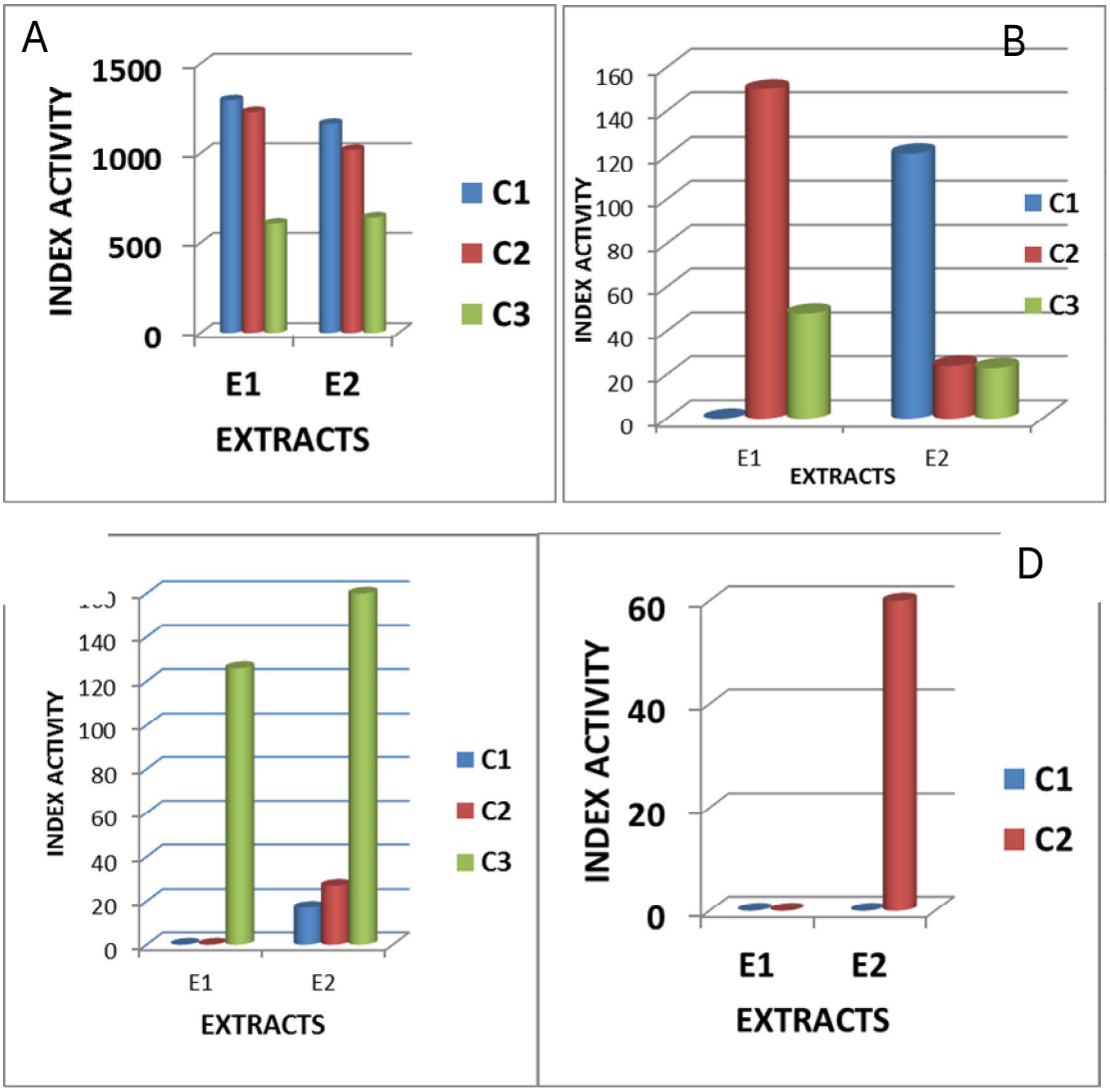

Figure 1: The activity index of *Combretum hereroense* (E1) and *Canthium mundianum* (E2) on the expression of IL-4 from three HIVnegative (A, B and C) individuals and one (D) HIVpositive donor at three different plant concentrations < C1= 300µg/ml, C2= 50µg/ml and C3= 20µg/ml >.

Immunocompromised donor (E) showed a positive impact at higher concentration of 300µg/ml only and was inhibited at 50µg/ml, whereas donor (F) showed the opposite of (E) in figure 2.

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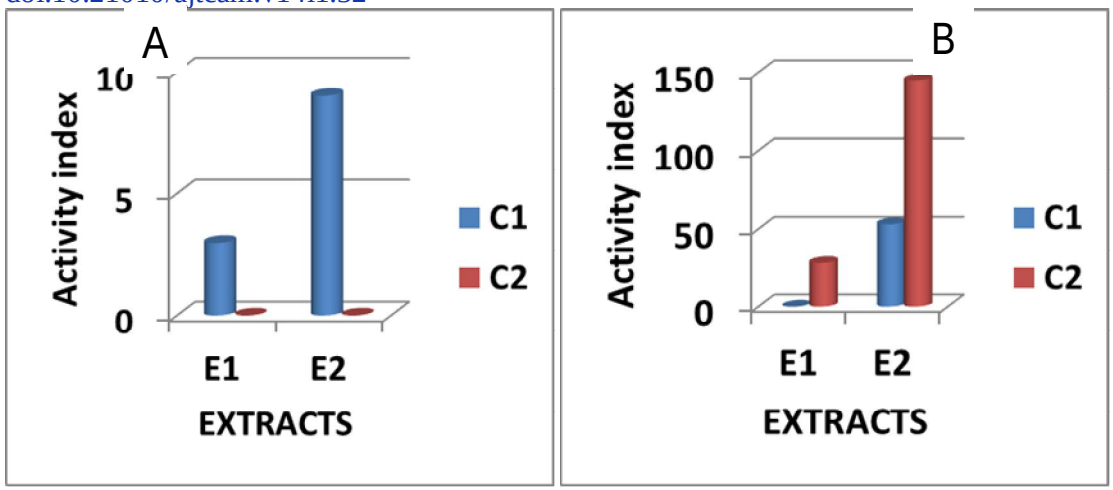
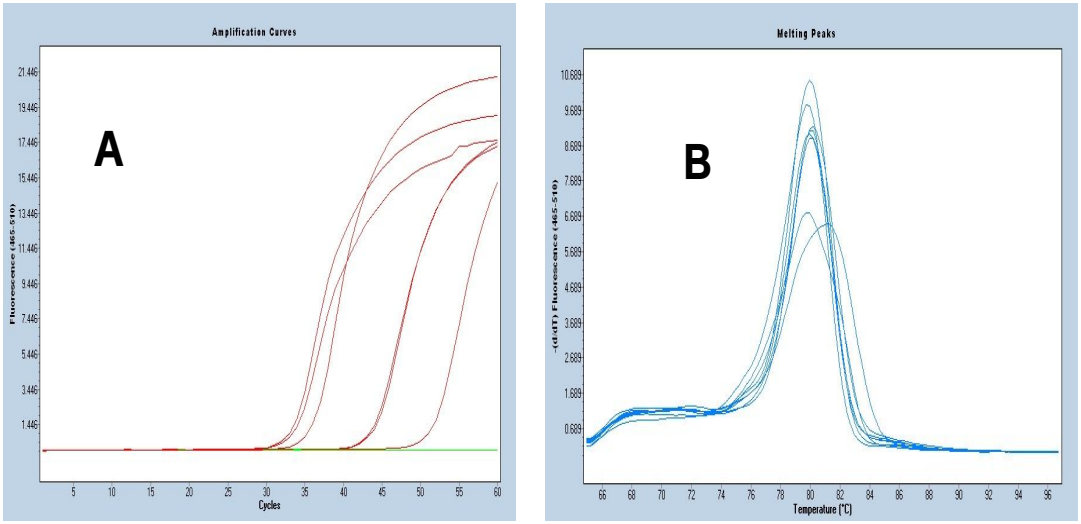


Figure 2: The activity index of *Combretum hereroense* (E1) and *Canthium mundianum* (E2) on the expression of IL-4 from two immunocompromised donor (F and F) at different concentrations of the plant extracts < C1= 300µg/ml, C2= 50µg/ml>.

Evaluation of IL-4 gene expression by reverse transcriptase real -time PCR

The amplification of human IL-4 gene through the detection of mRNA by reverse transcriptase real time PCR generated amplification curves and the melting curves as shown in **Figure 3**. Expression levels of interleukin-4 were determined for each HIV positive patient. All samples were run for 60 cycle and all sharp peaks suggested the sample which were amplified by the primers used.



Figures 3: Examples of amplification curves (A) and melt curves (B) obtained from the amplification of the IL-4 cDNA from activated PBMCs exposed to the medicinal plant extracts from *Combretum hereroense* and *Canthium mundianum.*

The effects of medicinal plants on IL4 gene expression

Figure 4 shows the effect of the two plant extracts tested on the amplification of IL-4 mRNA for all immunocompromised individuals. This Figure indicates that the expression of IL-4 was higher for *Combretum hereroense* and was lower with *Canthium mundianum* and was dose dependent.

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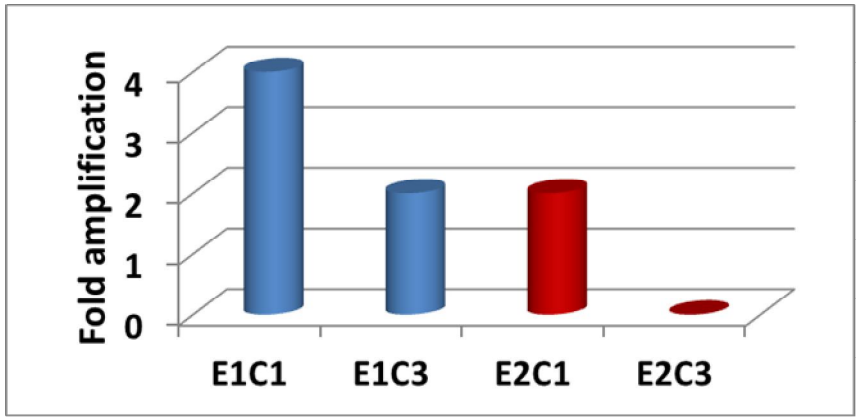

Figure 4: The effects of *Combretum hereroense* (E1) and *Canthium mundianum* (E2) on the gene expression of IL-4 from immunocompromised individuals: < C1= 300µg/ml, C3= 20µg/ml.

Discussion

This study was proposed to establish an in-vitro model for determination of the effects of plant extracts on IL-4 production and expression level using peripheral blood mononuclear cells. This is based on the fact that IL4 has been proven more than 20 years ago to be an anti-inflammatory cytokine (Woodward et al., 2010). Therefore one can estimate that a plant extract that increase the production of IL4 may have anti-inflammatory activity. Water extracts were used because water is the most commonly used extractant by traditional healers (Haidara et al 2016; Vasisht et al., 2016). The results of our study indicate that IL-4 is produced at different levels according to previous pathologic conditions of the blood donors in a dose dependent fashion. IL-4 is a highly pleiotropic cytokine that is able to influence Th cell differentiation and early secretion of IL-4 leads to polarization of Th cell differentiation toward Th2-like cells (Kaiko et al., 2007). It also decreases the production of Th1 cells, macrophages, INF-gamma, and dendritic cell. A study done by Amirghofran et al. (2011) showed that *Linum persicum* significantly inhibited both TNF-α and IL-1β cytokine production by stimulated macrophages. However, *D. kotschyi*, *D. termeana* and *F. angulata* decreased the secretion of IL-1β from the cells. Similarly, the present study showed differences in the inhibition or the secretion of IL-4 in both immunocompetent and immunocompromised individuals. Although we discovered that the level of IL-4 production is inversely proportional to the concentration mostly for HIV infected donors (the higher the concentration the lower the level of IL-4 production and expression), this effect varied from individual to individual as it was observed in one of the three HIV negative individuals. This is an indication that such increase could be associated with previous or existing infections. For example, previous studies have shown that the increase in the IL-4 production was found associated with leptospirosis (Koth and Clandra., 2003).

In HIV negative individuals the level of IL-4 production was proportional to the concentration of the extract, the higher the concentration the higher the production and expression of IL-4. Such reactions have been associated with secondary reaction such as skin reactions among patients who have taken plant extracts (Norisugi et al., 2014). From previous studies multiple mechanisms have been suggested to explain the progressive production of cytokines. It has been reported that phytohemaglutinin (PHA) induced production IL-2 and INFγ known as T-helper type 1 (TH1) cytokines, by peripheral blood mononuclear cells (PBMC) decreases with progression of disease, whereas IL-4 production, a Th2 cytokine increases with HIV disease progression (Kedzierska and Crowe, 2001). Rao et al. (2008) showed that IL-4 exerts both stimulatory and inhibitory effects on HIV-1 replication depending on the stage of maturation of monocytes into macrophages.

Kothari and Deshmukh (2006) reported that increase in the production of IL-4 was found to be associated with a Th2 cytokine profile although this action also lead to the modulation of Th1 response with increased production of Interferon gamma (Fukao et al., 2000). The outcome of an increase production of Interleukin 4 is highly variable and may depend on several factors such as infection history of the donor, the concentration of other cytokines such as IL12 and the physiological conditions of the donor such as in the case of diabetic patients (Li et al., 2014). This activation could lead to remission of infections as described by Li et al., (2014) or to a remission of allergic reactions as described by Machado et al., (2015). However, other authors such as Valentin et al., (1998) have demonstrated that the increased production of IL4 in HIV patients could lead to an increase formation of syncytia-inducing from non-cyncytia-inducing HIV1 with an accelerated disease progression as a result. In our study, a very low level of IL-4 production was detected in both HIV infected donor and one HIV uninfected donor. This is a possible indication of the potential positive action of the extracts on disease progression of HIV. A similar finding to these, were previously reported (Gilliland et al., 1990). Previous findings favor the hypothesis that increased production of IL-4 is not a marker of the Th2 patterns during HIV disease progression (Clerici and Shearer, 1993; Kothari and Deshmukh, 2006). In our study, there was a

general increase in the production of IL-4 mRNA more so in HIV positive individuals although this did not correlate with expression in terms of cytokine production as measured by ELISA. In fact Real-time PCR is a highly sensitive technique enabling amplification and quantification of a specific nucleic acid sequence with detection of the PCR product. RT – PCR demonstrated that IL-4 was increasingly produced by *Combretum hereroense* at high concentration and by *Canthium mundianum* at lower concentration for HIV+ donors. Considering that IL-4 is a cytokine that is mainly produced by Th2 cells (Storni et al., 2005) the increase in the production of IL-4 may result in an improvement or increase of the Th2 response and consequently leading to an increase in mounting an effective humoral and cell-mediated responses that are required to fight extracellular microbes and parasites (Fukao et al., 2000;Huang et al., 2016). It is however important to notice the variations related to the dosage further emphasizing the importance of the dosage in the management of different diseases.

A study on the immunomodulatory effects of the *Aloe vera* peel extract in splenocyte cultures, following stimulation by concanavalin A, showed that the extract did not affect the production of interleukin IL-2 and interferon-gamma but did promote the production of IL-4 and IL-10 (Kwon et al., 2009). It was suggested that the effect of *Aloe vera* extract could be due to emodin which has an immunomodulatory effect by promoting Th2 cytokines and reducing Th1 cytokines (Liu et al., 2009). In the present study, we observed similar results where *Combretum hereroense* and *Canthium mundianum* stimulated production and expression of IL-4 in both HIV- and HIV+ individuals; however, we were unable to identify the chemical compounds responsible for the effects observed. Overall our study indicated that *Combretum hereroense* and *Canthium mundianum* were able to modulate the production of interleukin 4 depending on the dosage and the pathologic conditions of the individuals. Further studies are needed to characterize the exact conditions of patients that guided the variation in the production of IL-4 and also to confirm these activities in an *In vivo* model. The potential compounds responsible for these activities need to be identified as well.

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